CORE
Metadata, citation and similar papers at core.ac.uk
Provided by Elsevier - Publisher Connector

Dual effects of organic solvents on chloroplast phosphoribulokinase and NADP-glyceraldehyde-3-P dehydrogenase

Ricardo A. Wolosiuk, Esteban Corley, Nancy A. Crawford[+] and Bob. B. Buchanan[+]

Instituto de Investigaciones Bioquimicas, Facultad de Ciencias Exactas y Naturales (UBA), Antonio Machado 151, (1405) Buenos Aires, Argentina and [+]*Division of Molecular Plant Biology, University of California, Berkeley, CA 94720, USA*

Received 28 May 1985; revised version received 8 July 1985

Organic solvents miscible in water (cosolvents) exerted a dual effect on the activation stage of two thioredoxin-linked enzymes of the reductive pentose phosphate cycle, phosphoribulokinase and NADP-glyceraldehyde-3-P dehydrogenase, both from spinach chloroplast; the enzyme specific activity was stimulated and inhibited by low and high concentrations of alcohols, respectively. On the contrary, cosolvents inhibited the catalytic process. In the stimulation of phosphoribulokinase activation, organic solvents reduced the requirement for thioredoxin-f and changed the thiol specificity, so that monothiols became functional. The cosolvent-mediated enhancement of NADP-glyceraldehyde-3-P dehydrogenase was obtained in the absence of modulators. With both enzymes, the concentration of the organic solvents required for activation was inversely proportional to its hydrophobicity (1-butanol < 1-propanol < 2-propanol < ethanol). The present results demonstrate the participation of a new component, the enzyme microenvironment, in the regulation of thioredoxin-linked chloroplast enzymes.

Chloroplast enzyme *NADP-glyceraldehyde-3-phosphate dehydrogenase* *Phosphoribulokinase* *Organic solvent* *Enzyme activation* *Photosynthesis*

1. INTRODUCTION

Regulatory enzymes of the reductive pentose phosphate cycle of higher plant chloroplasts are considered to be soluble and, consequently, have typically been studied in vitro with water as the sole solvent. However, any interaction of these enzymes with thylakoid membranes indicates that their physiological environment is not strictly aqueous [1,2]. The use of mixtures of water and organic solvents opens the possibility to study these enzymes in an in vitro milieu that, although unphysiological, resembles a membrane environment in being less polar than water [3]. Such an approach seems attractive in the case of regulatory enzymes of the reductive pentose phosphate cycle because their activity is due to two reactions, activation and catalysis, that can be separated experimentally and selectively modified [4–7]. The activation process has been shown to be controlled independently by a broad spectrum of agents: ions (H^+, Mg^{2+}, Ca^{2+}), cycle intermediates (sugar phosphates) and reductants (reduced thioredoxin, NADPH) [8–13]. The activation reaction would thus seem to be an ideal site for organic solvent effects vis-a-vis its catalytic counterpart.

A recent report shows that fructose-1,6-bisphosphatase, a thioredoxin-f-linked enzyme of chloroplasts, is influenced by cosolvents [14]. Dependent on their concentration, organic solvents either enhanced or inhibited the concerted activation of the enzyme. The stimulation of the specific activity of fructose-1,6-bisphosphatase brought about by cosolvents resulted from a decrease in the activation kinetic constants of modulators (Ca^{2+}, fructose-1,6-bisphosphate). In

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/85/$3.30 © 1985 Federation of European Biochemical Societies

contrast, cosolvents inhibited catalysis, i.e. the hydrolysis of fructose-1,6-bisphosphate by the activated enzyme.

To determine whether other thioredoxin-linked chloroplast enzymes show a response to cosolvents, we have determined the effects of organic solvent mixtures on the activity of phosphoribulokinase and NADP-glyceraldehyde-3-P dehydrogenase, 2 regulatory enzymes of the reductive pentose phosphate cycle, which can weakly interact with thylakoid membranes [1,2]. We now report that each of these enzymes is influenced by environments less polar than water in a manner generally similar to fructose-1,6-bisphosphatase.

2. MATERIALS AND METHODS

2.1. *Reagents*

Biochemicals and auxiliary enzymes were obtained from Sigma (St. Louis, MO). Other chemicals were purchased from commercial sources and were of analytical grade.

2.2. *Purification of chloroplast enzymes and thioredoxin-f*

Previously devised procedures were followed for the purification of the regulatory forms of both phosphoribulokinase [12] and NADP-glyceraldehyde-3-P dehydrogenase [13] from spinach leaves. The procedures in [15] were used for the purification of thioredoxin-f and the estimation of protein.

2.3. *Assay of enzyme activities*

Determination of enzyme activity was carried out by the 2-stage assay used for the study of hysteretic enzymes [16]. Following preincubation of the enzyme at 23°C in a mixture of specific composition (see below), catalytic activity was measured spectrophotometrically by injecting the enzyme into the assay solution and measuring change in absorbance at 340 nm.

2.3.1. Phosphoribulokinase

The assay solution for phosphoribulokinase contained 2.5 units of rabbit muscle lactate dehydrogenase; 2.1 units of rabbit muscle pyruvate kinase; 3.0 units of phosphoribose-isomerase and the following: 50 μmol Tris-HCl buffer (pH 7.9); 10 μmol $MgSO_4$; 10 μmol KCl; 0.8 μmol sodium phospho*enol*pyruvate; 1.0 μmol ATP; 0.2 μmol NADH; and 0.4 μmol sodium ribose-5-P. Final volume: 1.0 ml. The decrease in absorbance at 340 nm was measured.

2.3.2. NAD(P)-glyceraldehyde-3-P dehydrogenase

The assay solution for the NAD(P)-linked activity of glyceraldehyde-3-P dehydrogenase contained 2 units of yeast phosphoglyceric-phosphokinase and the following: 50 μmol Tris-HCl buffer (pH 7.9); 10 μmol $MgSO_4$; 2.5 μmol sodium 3-phosphoglycerate; 2.5 μmol ATP; and 0.2 μmol NAD(P)H. Final volume: 1.0 ml. The decrease in absorbance at 340 nm was measured.

3. RESULTS AND DISCUSSION

Following chromatography on a Bio-Gel A-1.5 m column, spinach leaf preparations showed 2 peaks of phosphoribulokinase activity, corresponding to the regulatory and nonregulatory forms of the enzyme [12]. As seen in table 1, the regulatory form was strongly activated by preincubation with dithiothreitol-reduced thioredoxin-f. When activation was carried out in a solution

Table 1

Effect of 2-propanol on the activation of phosphoribulokinase

Preincubation conditions	Phosphoribulokinase activity (nmol NADH oxidized · min^{-1})	
	Control	2-Propanol
None	0	0
Dithiothreitol	8	28
Dithiothreitol plus thioredoxin	30	32

The partially purified form of phosphoribulokinase (5 μg protein) was preincubated at 23°C in 0.1 ml of a solution containing 10 μmol Tris-HCl buffer (pH 7.9) and, as indicated, 0.5 μmol dithiothreitol, 2 μg thioredoxin-f and 10% 2-propanol. After 5 min of preincubation, an aliquot of the mixture (0.05 ml) was injected into the solution for assaying phosphoribulokinase activity described in section 2

Table 2

Effect of ethanol on the activity of cosolvent-activated phosphoribulokinase

	Phosphoribulokinase activity (nmol NADH oxidized · min^{-1})
Control	54
Plus 2% ethanol	50
Plus 5% ethanol	18
Plus 10% ethanol	1

The regulatory form of phosphoribulokinase (5 μg protein) was preincubated at 23°C in 0.1 ml of a solution containing 10 μmol Tris-HCl buffer (pH 7.9), 0.5 μmol dithiothreitol and 20% ethanol. Following 5 min of preincubation, an aliquot (0.03 ml) of the mixture was injected into the solution for assaying phosphoribulokinase activity described under section 2

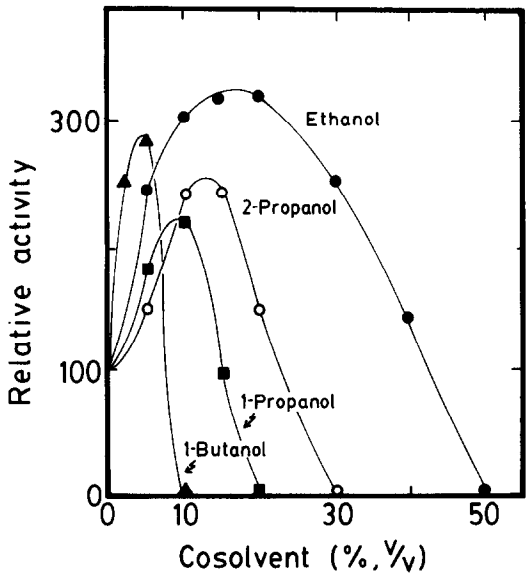

Fig.1. Effect of organic solvents on the activation of phosphoribulokinase. The partially purified regulatory form of phosphoribulokinase (5 μg protein) was preincubated at 23°C for 5 min in 0.1 ml of a solution containing 10 μmol Tris-HCl buffer (pH 7.9), 0.5 μmol dithiothreitol and, as indicated, the organic solvent. After preincubation, an aliquot (0.025 ml) of the mixture was injected into the solution for assaying phosphoribulokinase activity described in section 2. Control activity: 15 nmol NADH oxidized · min^{-1}.

containing 10% 2-propanol, the enzyme was activated by dithiothreitol in the absence of thioredoxin-f. Since our experimental protocol permits a separation of enzyme activation from catalysis, we determined the effect of 2-propanol on the catalytic reaction. In those studies (table 2), we found that 2-propanol consistently inhibited catalysis even at 10% or lower concentrations. In parallel experiments, we observed that concentrations of 2-propanol lower than 10% did not inhibit the activity of the couple pyruvate kinase/lactate dehydrogenase and ribose 5-P isomerase. Thus, 2-propanol and other alcohols (see below) stimulated the conversion of phosphoribulokinase from an inactive form to another more active, as found earlier for activation by reduced thioredoxin-f [12]. On the other hand, the nonregulatory form of phosphoribulokinase, which does not respond to thioredoxin-f [12], was inactivated by organic solvents (not shown).

To further characterize the effect of cosolvents, we studied activation of the regulatory form of phosphoribulokinase by different alcohols added in the presence of dithiothreitol. As shown in fig.1, the concentration of cosolvent required for maximal enhancement of specific activity decreased as a function of chain length – i.e., butanol < propanol < ethanol. An examination of the octanol-water partition coefficient for the alcohols tested [17] suggested that the enhancement of phosphoribulokinase activation by organic solvents in the presence of dithiothreitol correlates with the hydrophobic nature of the cosolvent.

To assess the role of thiols in the activation of phosphoribulokinase by aliphatic alcohols, we replaced dithiothreitol by other sulfhydryl compounds added in the absence of thioredoxin-f. Table 3 illustrates that, while only dithiols showed significant enhancement of phosphoribulokinase activity in an aqueous medium, monothiols became effective following a 5 min exposure of the enzyme to 20% ethanol. The results thus show that in addition to enhancing the dithiol-mediated activation of phosphoribulokinase, organic solvents added during activation changed reductant specificity so that the requirement for thioredoxin-f was reduced and monothiols became effective.

To determine whether other thioredoxin-f-linked enzymes of chloroplasts are similarly modified, we tested the effect of organic solvents

Table 3

Effect of ethanol on the thiol-mediated activation of phosphoribulokinase

Preincubation conditions	Phosphoribulokinase activity (nmol NADH oxidized · min^{-1})	
	Control	Ethanol
None	0	0
Dithiothreitol	16	56
1,3-Dimercapto-2-propanol	16	64
2-Mercaptoethanol	0	32
2-Mercaptoacetate	3	16
L-Cysteine	3	16
Reduced glutathione	2	28

The regulatory form of phosphoribulokinase (5 μg protein) was preincubated at 23°C in 0.1 ml of a solution containing 10 μmol Tris-HCl buffer (pH 7.9) and, as indicated, 20% ethanol and either 0.5 μmol of a dithiol or 1.0 μmol of a monothiol. Following 5 min preincubation, the mixture was injected into the solution for assaying phosphoribulokinase activity described under section 2

on glyceraldehyde-3-P dehydrogenase. As illustrated in fig.2, the NADP-linked activity of the preparation was enhanced by preincubating the enzyme with a cosolvent. Under similar conditions, the NAD-linked activity was unaffected (not shown). Although the concentrations of cosolvent necessary for maximal enhancement of the specific activity were higher with NADP-glyceraldehyde-3-P dehydrogenase in comparison with the other enzymes tested (cf. fructose-1,6-bisphosphatase in [14] and phosphoribulokinase in this paper), its behavior was similar in that lower concentrations of organic solvents were required as their hydrophobic character increased. In other experiments (not shown), it was found that cosolvents enhanced the activation of glyceraldehyde-3-P dehydrogenase by P_i [13].

In conclusion, our earlier studies [14] and the present results indicate that transition from a strictly aqueous milieu to an environment less polar than water differentially influences thioredoxin-f-linked enzymes of chloroplasts. With the enzymes studied so far (fructose-1,6-bisphosphatase, phosphoribulokinase, NADP-glyceraldehyde-3-P dehydrogenase), the addition

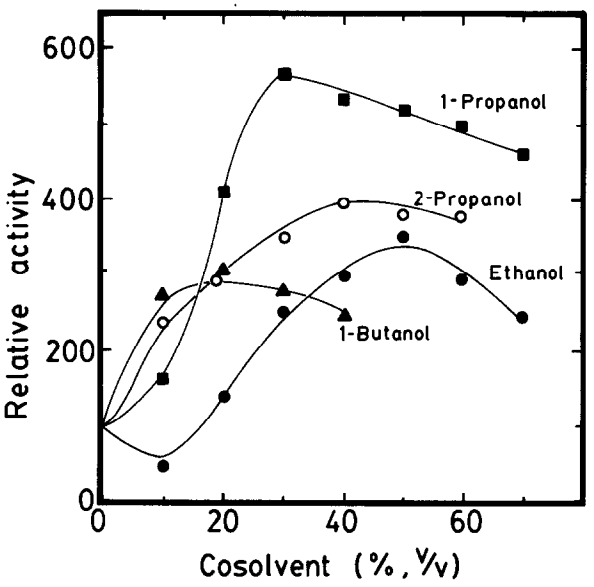

Fig.2. Effect of organic solvents on the activation of the regulatory form of NADP-glyceraldehyde-3-P dehydrogenase. The enzyme (6 μg protein) was preincubated at 23°C in 0.1 ml of a solution containing 10 μmol Tris-HCl buffer (pH 7.9) and, as indicated, the organic solvent. Following 5 min of preincubation, the mixture was injected into the solution for assaying the NADP-linked activity of glyceraldehyde-3-P dehydrogenase described in section 2. Control activity: 5 nmol NADPH oxidized · min^{-1}. When the concentration of *n*-butanol in the activation stage was higher than 10%, the mixture separated into 2 phases; for assaying enzyme activity we mixed thoroughly before injection.

of cosolvents to the activation process enhanced the specific activity, whereas cosolvents added during catalysis lowered the rate of substrate conversion. As these enzymes would interact with thylakoid membranes, and therefore function in a microenvironment different to bulk water, it becomes of interest to determine the relevance of the observed cosolvent effects to enzyme function in vivo. Apropos this point, it should be noted that in 2 of the cases studied (fructose-1,6-bisphosphatase, phosphoribulokinase) the cosolvent diminished the requirement for thioredoxin-f, and monothiols became effective as enzyme activators. In the third case (NADP-glyceraldehyde-3-P dehydrogenase), activation by cosolvents did not require thiols. Interestingly, a somewhat different pattern has taken shape with NADP-malate

dehydrogenase, a chloroplast enzyme activated by either thioredoxin-m or thioredoxin-f [19]. In this case, cosolvents have been found to enhance activation in a manner similar to the other enzymes, but a thioredoxin was still strictly required and monothiols remained ineffective in activation [20]. It remains to be seen how each of these enzymes (which are regulated by the ferredoxin/thioredoxin system which solubilized in aqueous solution) respond to thioredoxins and effectors provided in a physiological membrane system.

ACKNOWLEDGEMENTS

We are indebted to Dr Luis F. Leloir and other members of the Instituto de Investigaciones Bioquimicas for discussions and criticism. R.A.W. is a career investigator of the Consejo Nacional de Investigaciones Cientificas y Tecnicas (CONICET-Argentina). This work was aided by a cooperative research grant from the National Science Foundation (USA) and CONICET (Argentina) (Proyecto 042), as well as by grants from both the Metabolic Biology Program of the National Science Foundation and CONICET to B.B.B. and R.A.W., respectively.

REFERENCES

[1] Fischer, K.H. and Latzko, E. (1979) Biochem. Biophys. Res. Commun. 89, 300–306.
[2] Anderson, L.E. and Ben-Bassat, D. (1981) in: Photosynthesis (Akoyunoglos, G. ed.) vol.4, pp.373–378, Balaban International Science Services, Philadelphia.
[3] Tan, K.H. and Lovrien, G. (1972) J. Biol. Chem. 247, 3278–3285.
[4] Anderson, L.E. (1979) in: Photosynthesis II, Photosynthetic Carbon Metabolism and Related Processes, Encycl. Plant Physiol. (Gibbs, M. and Latzko, E. eds) vol.6, pp.271–281, Springer, Berlin.
[5] Buchanan, B.B., Wolosiuk, R.A. and Schurmann, P. (1979) Trends Biochem. Sci. 4, 93–96.
[6] Huber, S. (1982) in: Cellular and Subcellular Localization in Plant Metabolism (Creasy, L.L. and Hrazdina, G. eds) pp.151–184, Plenum, New York.
[7] Kelly, G.J. and Latzko, E. (1982) Progr. Bot. 44, 103–131.
[8] Lorimer, G.H., Badger, M.R. and Andrews, T.J. (1976) Biochemistry 15, 529–536.
[9] Wolosiuk, R.A. and Buchanan, B.B. (1977) Nature 266, 565–567.
[10] Schurman, P. and Wolosiuk, R.A. (1978) Biochim. Biophys. Acta 522, 130–138.
[11] Hertig, C.M. and Wolosiuk, R.A. (1983) J. Biol. Chem. 258, 984–989.
[12] Wolosiuk, R.A. and Buchanan, B.B. (1978) Arch. Biochem. Biophys. 189, 97–101.
[13] Wolosiuk, R.A. and Buchanan, B.B. (1976) J. Biol. Chem. 251, 6456–6461.
[14] Corley, E. and Wolosiuk, R.A. (1985) J. Biol. Chem. 260, 3978–3983.
[15] Wolosiuk, R.A., Crawford, N.A., Yee, B.C. and Buchanan, B.B. (1979) J. Biol. Chem. 254, 1627–1632.
[16] Wolosiuk, R.A. and Hertig, C.M. (1983) in: Thioredoxins, Structure and Functions (Gadal, P. ed.) pp.167–173, Editions du CNRS, Paris.
[17] Helmer, F., Kiehs, K. and Hansch, C. (1968) Biochemistry 7, 2858–2863.
[18] Butler, L.G. (1979) Enzyme Microb. Technol. 1, 253–259.
[19] Schurmann, P., Maeda, K. and Tsugita, A. (1981) Eur. J. Biochem. 116, 37–45.
[20] Crawford, N.A., Yee, B.C., Hutchenson, S.W., Wolosiuk, R.A. and Buchanan, B.B., submitted.